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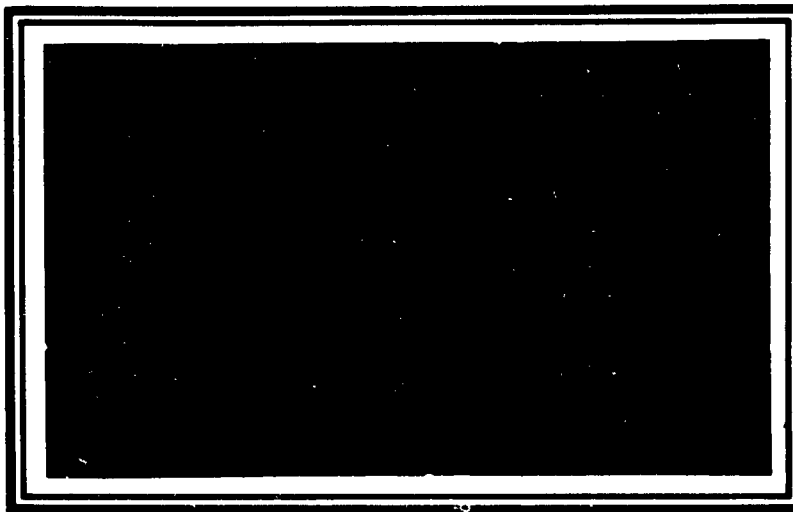
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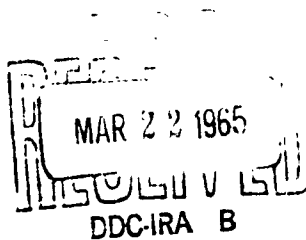
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TECHNICAL REPORT

U. S. NAVAL APPLIED SCIENCE LABORATORY

NAVAL BASE
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REARING BARNACLE CYPRIDS IN THE LABORATORY
FOR MARINE FOULING STUDIES

SR 007-08-05, Task 1201

Lab. Project 9300-22, Progress Report #1

19 MAR 1965

A. FREIBERGER

MATERIAL SCIENCES DIVISION
D. H. KALLAS, Head

Approved: 

E. J. JENNE
Technical Director

Approved: 

I. F. PIKE, Capt. USN
Commanding Officer & Director

U. S. NAVAL APPLIED SCIENCE LABORATORY
NAVAL BASE
BROOKLYN, NEW YORK 11251

ABSTRACT

Studies of marine fouling and evaluations of antifouling coatings and methods have been carried on at field stations under naturally occurring conditions. A more aggressive, fundamental study of the marine-fouling problem can be attempted, and evaluations of antifouling materials can be accomplished with greater speed and regularity, if studies employing live marine organisms are conducted under laboratory-controlled conditions. The techniques which, for the first time, succeeded in the laboratory mass-rearing for four species of acorn barnacles indigenous to the U. S. Atlantic coastline are set forth. The methods for removal of embryos from parent barnacles, feeding, and maintenance of proper environment for larval stages are described. Methods are described by which fertile barnacle ova are hatched, and metamorphose with successive ecdyses through six free-swimming naupliar stages, to the swimming-and-crawling cyprid stage. Photomicrographs of barnacle embryos, the six naupliar larval stages, and the presettled cyprid larval stage are presented. The future capabilities of this laboratory methodology and its anticipated contribution toward the development of improved anti-fouling materials are discussed.

SUMMARY

In order to subject the problem of marine fouling control to systematic scientific inquiry, means for studying the settlement, attachment and growth of fouling organisms are required. Requisite to such studies is a controlled supply of presettled larvae of the organisms under investigation. In this report, the techniques involved in the acquisition, identification and embryo-stripping of adult barnacles; and the hatching and rearing of barnacle larvae to the presettled (planktonic) cyprid stage are set forth. The cyprid is the stage at which barnacles settle and attach to submerged marine surfaces, and as such will be used as the organism in a bio-assay methodology for studying marine fouling and antifouling agents. Barnacle larvae of species Balanus amphitrite, B. eburneus, B. improvisus, and B. trigonus have been successfully mass-reared to the cyprid stage at this Laboratory at a location distinctly removed from their natural habitats. Vigorous, healthy larvae cultures have been maintained upon a diet of the diatom Cyclotella nana. Preliminary techniques for the controlled settlement and attachment of cyprids to test antifouling panels have been developed.

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ADMINISTRATIVE INFORMATION

- Ref: (a) NAVAPLSCIENLAB Program Summary, Subproject SR-007-08-05, Task 1201 of 1 Nov 1964
(b) NAVAPLSCIENLAB Project 9300-22 Tech Memo #1 of 25 May 1964
(c) NAVAPLSCIENLAB Project 9300-22 Tech Memo #3 of 8 Jul 1964
(d) NAVAPLSCIENLAB Project 9300-22 Tech Memo #2 of 17 Jun 1964

1. The development of procedures for studying marine fouling in the Laboratory, authorized by reference (a), is continuing at the U. S. Naval Applied Science Laboratory. The current work on this program concerns, mainly, the development of techniques for the mass rearing of juvenile barnacles, from embryo to the planktonic cyprid stage, under controlled laboratory conditions; and studies of the means for use of barnacle cyprids with a laboratory methodology for evaluating the effectiveness of antifouling coatings.

ACKNOWLEDGMENT

2. This investigation was conducted under the direction of Mr. H. Lacks, Head of the Coatings Branch. The laboratory work on rearing barnacle larvae was performed, mainly, by Mr. C. P. Cologer, with the assistance of Mr. G. Liberatore of the staff of the New York Aquarium. Dr. R. F. Nigrelli, Director of Research for the New York Zoological Society, served as technical consultant on problems of marine biology. The support of the Bureau of Ships Program Manager, Mr. E.A. Bukzin (Code 342A), and the Bureau's Coatings Unit Head, Mr. L. S. Birnbaum, is acknowledged.

INTRODUCTION

3. Fouling results from the growth of animals and plants on the surfaces of submerged objects. The growth of fouling organisms on ships results in corrosion of ships' hulls, increased fuel consumption due to increased frictional resistance to the movement of the hull through the water, losses in time and money in applying the necessary remedial measures, and reduction in the efficiency of underwater sound devices. The problem of combatting the fouling of ships has been the subject of intensive study since ancient times, and written records of the treatment of ships' bottoms dating from the 5th century B.C. have been found. It is only within fairly recent times, however, that means to combat marine fouling have been subject to systematic scientific inquiry. (1)* Antifouling paints, some of them quite successful at discouraging

* Note: The numbers in parentheses (in the text) refer to the Bibliography at the end of this report.

the attachment and growth of marine organisms for as long as two years under certain conditions, have been formulated, and are evaluated in harbor test stations or on ships at sea. Fouling is still a problem, however. The effective life of antifouling paints is limited. Costs involved in taking vessels out of service for removal of fouling accumulations and reapplication of antifouling paints are very high, probably in the neighborhood of a quarter of a billion dollars annually in the United States alone. The improvement of antifouling paints is a continuing process; in recent years new high-potency toxics, antimetabolic chemicals, and other biological excitatory or inhibitory substances have become available which may provide new approaches to fouling control. Efficient study of the large numbers of antifouling formulations suggested by the advent of these new materials requires new methods for study and evaluation that will provide prompt answers about the precise mode of antifouling action and that will possess far more control than the currently accepted procedures of protracted, non-controllable natural fouling in harbor test sites.

4. The object of the Naval Applied Science Laboratory's studies in the field of marine fouling is the development of a laboratory methodology for studying and evaluating antifouling coating systems; with the ultimate goal being the development of improved materials and processes for control of marine fouling. This report is concerned, mainly, with a description of the laboratory techniques, evolved and developed by this Laboratory, for the successful laboratory mass-rearing of barnacle cyprids.

5. Barnacle larvae, following hatching from fertilized ova, undergo a series of metamorphoses and pass through several stages of development. (See Appendix B.). The first six (in the case of the species currently being reared) of these stages are called naupliar stages, the larvae being known as nauplii (singular: nauplius). This nauplius is a free-swimming micro-organism ranging in size from about 200 to 800 microns. The sixth-stage nauplius metamorphoses into a swimming-and-crawling larval stage called a cyprid. It is the barnacle cyprid which ultimately settles on a submerged surface, attaches itself thereto, builds a calcareous shell, and becomes "fouling". The availability of quantities of barnacle cyprids, as supplied by techniques described herein, will provide the basis for a bio-assay technique for measuring properties of antifouling coatings with respect to a biological response.

BACKGROUND

6. This Laboratory is approaching the study of fouling control in a way that will permit close observation of the factors involved in fouling, provide laboratory surveillance of the fouling organisms at all stages of their

development from embryo to adult, and provide the means for observation and study of mechanisms which lead to attachment. This approach is based upon the development of methods for rearing large quantities of barnacle larvae of several species in the laboratory, with irregularities, which inevitably attend experiments dealing with living organisms, kept to a minimum. The primary requisite of the Naval Applied Science Laboratory's methodology is a steady supply of vigorous, healthy barnacle cyprids. Although there is presently no known, working laboratory methodology for studying fouling control which is completely reliable, which operates under precisely defined standards, and which can be relied upon to quickly provide repeatable data about the anti-fouling properties of coatings, attempts to evaluate antifouling coatings and toxic extracts of antifouling materials by observation of their effect upon laboratory reared larvae have been described by Tada (2) and Ragg (3). Studies of the developmental stages of several species of barnacle larvae, collected, mainly, from samples of naturally occurring plankton, have been carried on by Knight-Jones and Waugh (4), Barnes and Barnes (5), Jones and Crisp (6) and others. Small-scale cultures of juvenile barnacles which provided the larvae for anatomical and morphological studies are described by Bassindale (7), Costlow and Bookhout (8), Wisely (9) and Barnes and Costlow (10). Moyse (11) succeeded in mass rearing juveniles of the species Elminius modestus, and briefly describes the rather simple procedures and techniques employed. These procedures were employed during preliminary investigations of barnacle rearing by this Laboratory, but were found to be unsuccessful in connection with the species being reared, and resulted in 100% mortality at the third naupliar stage. A more detailed account of feeding laboratory-reared barnacle larvae of several species by Moyse (12), reports on the types and concentrations of algae which were used and provides information on the time of larval development from ova to cyprid for Balanus balanoides, Elminius modestus, Chthamalus stellatus and Lepas anatifera.

7. The general approach to fouling studies being pursued by this Laboratory, and an outline of the methodology being developed were reported in references (b) and (c), and set forth by Freiburger and Cologer (13). Briefly, the Naval Applied Science Laboratory methodology for studying and evaluating marine antifoulants consists of the following steps:

- a. Collection, from natural sources, of adult barnacles.
- b. Removal of embryos from the adult.
- c. Rearing the juvenile barnacles through the successive naupliar and planktonic cyprid stages.
- d. Exposure of antifouling test panels to a fixed number of planktonic cyprids under controlled conditions.
- e. Determination of the number of cyprid attachments, or observation of the vigor of the shell-building capability of the young attached barnacles.
- f. Interpretation of the "attachment data" as an indication of service antifouling effectiveness.

Reference (d) reported, briefly, on this Laboratory's initial success in mass rearing barnacle larvae to the planktonic cyprid stage, and provided preliminary results of observations of cyprid settling and attachment.

METHODS

8. The techniques and equipment described below are currently being employed to mass-rear barnacle larvae, from the fertilized ova to the planktonic cyprid stage, at this Laboratory's marine fouling test facility located, in association with the marine biological research laboratories of the New York Zoological Society, in the Aquarium at Seaside Park, Brooklyn, New York. The stepwise procedures for the Laboratory rearing of barnacle cyprids may be listed as follows:

- a. Collection, from natural sources, of embryo-bearing adult barnacles.
- b. Delivery of adult barnacles to the laboratory.
- c. Identification by species of adult barnacles.
- d. Removal of lamellae (masses of fertile ova) from the adult barnacles.
- e. Classification of degree of maturity of the lamellae.
- f. Hatching of fertile ova (embryos) to first stage nauplii and metamorphosis to second stage nauplii.
- g. Examination and count of nauplii, and transfer to rearing vessel.
- h. Development of nauplii, through successive metamorphoses to the third, fourth, fifth and sixth naupliar stages, and the planktonic cyprid stage.

9. A description of the methods for the collection of embryo-bearing adult barnacles from natural sources at Biscayne Bay, Florida, and their shipment by air-freight to the Laboratory; and a description of the source and treatment of the natural seawater used at the marine fouling test facility was given in reference (b). Except as otherwise noted or described in detail below, the methods of reference (b) are used in connection with current rearing techniques.

10. Identification of Adult Barnacles and Removal of Lamellae

The techniques described herein are currently being used to successfully mass-rear larvae of the following species of acorn barnacles: Balanus amphitrite, B. eburneus, B. improvisus and B. trigonus. The adult barnacles

of these species are readily identifiable by the color, shape and markings of their calcareous shell plates. (This is described in greater detail in reference (b), a portion of which is reproduced herein as Appendix C). In the Laboratory, the larvae of the various species are reared separately; each rearing vessel containing juvenile barnacles of only one species. The adult barnacles, usually measuring about 10 to 20 mm at the base, are removed from the aluminum panels upon which they have grown with the use of a sharp-edged spatula. When properly done, the basal plate of the barnacle's calcareous shell remains attached to the panel and the animal is thus completely exposed. Should the basal plate part from the surface of the panel and remain with the barnacle, the plate is carefully pried away with a forceps. Embryos, when present, are contained in two lamellae, each consisting of from 300 to 1500 embryos enclosed within a jelly-like matrix, located laterally within the body sac of the barnacle. This sac is cut and drawn aside to expose the lamellae which may then be removed with either a dissecting needle or a pipette. (See Figure 5)

11. Classification of Lamellae

For the purposes of this investigation, the Laboratory has established a classification of barnacle lamellae in accordance with the maturity of the constituent embryos. Lamellae are thus classified in one of three groups, as follows:

a. Group 1 - These are the least developed (youngest) embryos. The lamella is white or light tan; the embryos, closely packed within the lamella, do not have the nauplian eye spot. Microscopic examination (200X) does not reveal any motion by individual embryos. Hatching to first stage nauplii will commence in about 3 days to 2 weeks.

b. Group 2 - The color of the lamella varies from light tan or pink to dark brown or red, depending upon the species and the age of the embryos. The embryos, still contained within an intact lamella, each have a characteristic nauplian eye spot. Microscopic examination reveals rhythmic motion of individual embryos at the periphery of the lamella. Hatching to first stage nauplii will commence in about 6 hours to 3 days.

c. Group 3 - These embryos have begun to separate. The lamella has ruptured and is no longer intact, and cannot be removed as an entity. The embryos are drawn off with the use of a pipette. Hatching to 1st stage nauplii is either completed or will take place within 6 hours. In general, group 3 lamellae are not used for this investigation.

12. Hatching

Immediately upon removal from the adult, the lamellae are placed in a Pyrex hatching beaker. Each hatching beaker may contain as many as eight lamellae, of

a given maturity group and of a single species of barnacle, in about 300 ml of filtered seawater maintained at $70^{\circ}\text{F} \pm 2$. (Unless otherwise noted, all seawater used for these studies is heated to $70^{\circ}\text{F} \pm 2$.) (The use of antibiotics at this stage of the rearing process, as reported in reference (c) and in (13), has been discontinued). As required by the observed rate of hatching, the contents of the hatching vessel are passed through a bolting-cloth sieve, specially designed and fabricated at the Laboratory for this purpose, into a Pyrex collecting vessel containing a quantity of filtered seawater. The methods for sieving populations of barnacle larvae at this Laboratory are patterned after procedures in use at the Department of Interior, Bureau of Commercial Fisheries Laboratory at Milford, Connecticut, for processing *Mullus* larvae (14). In this connection, the Fisheries Laboratory has found that commercially available stainless steel sieves are adequate for their purposes. For rearing barnacle nauplii as a bio-assay organism, however, this Laboratory has found it necessary to eliminate all contact with metals and to employ non-toxic bolting cloth for this purpose. The sieve, fabricated with No. 6, 74 mesh bolting cloth (size of openings: 343 microns), (See Figure 6) permits the passage of discrete embryos and free-swimming, newly-hatched first and second stage nauplii, each now measuring about 300 microns in length, and retains the lamellae masses of unhatched embryos which are returned to the hatching beaker.

13. Counting Nauplii

In order to separate vigorous, healthy larvae from more sluggish forms in the population of first and second stage nauplii, and to provide a count of the number of nauplii being reared, a transfer of larvae is made to a counting vessel. This transfer is facilitated by the fact that healthy nauplii are highly phototropic and will swim in the direction of a light source. By placing a small spotlight at the side of the collecting vessel so that a beam of light traverses the seawater horizontally, the nauplii can be made to concentrate at the side of the vessel nearest the spotlight. (See Figures 7 and 8). They may then be drawn off in great numbers with a pipette, and discharged into the counting vessel. In this manner a transfer of nauplii is made which leaves behind in the collecting vessel, for discard, the more sluggish, less vigorous larvae, as well as the empty ova cases, the exuviae (exoskeletal sheddings of the metamorphosis from first to second stage), the unhatched embryos, and any dead larvae which passed through the sieve. The volume of the seawater and nauplius population in the counting vessel is adjusted, with the addition of more filtered seawater, to 100 ml. The contents of the vessel is then mechanically agitated to evenly distribute the larvae, and a one-milliliter sample is removed with a pipette and placed in a well-slide.

The number of nauplii present in the one-milliliter sample is then counted under a microscope. This procedure is conducted four times and the results averaged and multiplied by 100 to obtain an approximate count of nauplii in the counting vessel. As a rule, larvae are further reared to the cyprid stage in populations numbering about 200. Therefore, the volume of seawater in the counting vessel which contains 200 nauplii is calculated on the basis of the total number of nauplii present in the 100 milliliters. This calculated volume, containing 200 first and second stage nauplii may then be measured out and transferred to a rearing vessel.

14. Rearing to Cyprid Stage

The following techniques and equipment are currently being employed for the mass-rearing of nauplii to the cyprid stage:

a. Rearing vessel - The rearing vessels, in which the development of nauplii to the cyprid stage takes place, consist of Pyrex beakers of 1500 ml capacity. A 4-inch diameter Pyrex Petri dish with a 1/2-inch inscribed grid, is placed in the bottom of each rearing vessel. (Note: It will be upon the surface of this Petri dish, which is placed in the rearing vessel at this early stage in order that it may develop a film of bacterial slime necessary for cyprid settlement, thus becoming "seasoned", that a preponderance of cyprids will ultimately settle and metamorphose into young adult barnacles. However, only those aspects of the techniques germane to rearing larvae to the cyprid stage are discussed herein. Laboratory studies of cyprid attachment and metamorphosis to adult will be the subject of future reports.) The following are then placed into the rearing vessel:

(1) A volume of seawater containing approximately 200 early stage barnacle nauplii of one species, prepared and counted as described in paragraph 11, above.

(2) A quantity of algae culture, calculated to contain 1.5×10^8 cells of the centric diatom Cyclotella nana. (The culturing of algae and the preparation of samples with known cell-counts are briefly described in Appendix D.)

(3) Filtered seawater to bring the total volume in the rearing vessel to 750 ml.

The rearing vessel is then loosely covered with a polyethylene bag and maintained at a temperature of $70^\circ\text{F} \pm 2$. (See Figure 9).

b. Maintenance and observation - The seawater in the rearing vessel is changed, ~~from~~ algae culture is added, and the progress of larval development is observed on a daily, seven-day-a-week basis. The steps for these procedures are as follows:

(1) The contents of the rearing vessel are passed through a sieve, fabricated with No. 20, 173 mesh bolting cloth (size of openings: 147 microns), which permits passage of the seawater, unused algae and most waste products, and retains the nauplii and the exuviae resulting from the ecdysis which occurs during metamorphosis from stage to stage. (See Figure 10). The rearing vessel and Petri dish are permitted to drain thoroughly, but are not rinsed or wiped dry. The larval population, now contained in the sieve, is gently flushed with running seawater and discharged into a shallow Pyrex examination dish together with sufficient seawater to maintain a depth of about 8 centimeters. (See Figure 11).

(2) The larval population is then examined under a microscope. The following information is derived from this examination:

(a) The larval stages present - The nauplii are observed ~~in vivo~~ and the stage or stages present are noted. Identification of naupliar stages is made in accordance with descriptions contained in the literature, (4) through (8) and others, and also based upon observation and enumeration of barnacle larvae development carried out at this Laboratory. The exuviae (shedded exoskeletons) are also examined. Since they are lifeless and do not move, they often facilitate the determination of the larval stages present in the population. For example, an exuvium identified as having belonged to a third stage nauplius, is indicative of larval development to the next higher, or fourth, naupliar stage.

(b) The predominating larval stage - A quantitative observation of the stages present is made, and the presence of a single predominating stage is noted, when applicable.

(c) General health and vigor - The swimming patterns and the overall appearance of the mechanical activity of the nauplii are observed as an indication of the health and vigor of the population.

(d) Evidence of feeding - As a general rule, it has been found that evidence of feeding provides a firm basis for establishing the health and development potential of a naupliar population. A nauplius is examined for the presence of diatoms in its gut, and for other internal signs that food is being assimilated. The presence and quantity of fecal pellets in the surrounding seawater are also noted as evidence of successful feeding.

(e) Dead nauplii - The presence of dead nauplii is noted, and a count of the number of living and dead forms is made in order to determine the overall percentage survival rate for the population.

(3) The contents of the shallow examining dish are then returned to the rearing vessel; a quantity of algae culture and of fresh seawater is added, as before; and the rearing vessel is again covered and maintained at $70F \pm 2$.

c. Appearance of Cyprids - When the larval population begins to metamorphose from the sixth naupliar stage to the free-swimming cyprid stage the following procedures take effect:

(1) Upon determination, during microscopic examination of a population contained in a shallow dish (as described in paragraph b. (2), above), that cyprids are present, the Petri dish which has resided within the rearing vessel is removed.

(2) The rearing vessel is thoroughly washed and scrubbed with running seawater.

(3) The Petri dish, drained, but unwashed and retaining any bacterial slime which may have formed upon its surface, is replaced in position at the bottom of the rearing vessel.

(4) The contents of the shallow examining dish are then returned to the rearing vessel; algae culture and fresh seawater are added, as before; and the rearing vessel is again covered and maintained at $70F \pm 2$.

15. Beginnings of Settlement, Attachment, and Metamorphosis to Adult Barnacle

The rearing vessel, constituted as described in paragraph 12, above, now consists of a 3/4-liter seawater environment containing a known number of healthy barnacle cyprids, and a 4-inch diameter "preseasoned", grid-scored, glass surface upon which, by reason of its bacterial-slime film, cyprid settlement may be expected to preferentially occur. Observations of cyprid settlement, attachment and metamorphosis to adult barnacle, are currently in progress at the Laboratory.

RESULTS

16. In the course of developing the techniques for the successful laboratory mass-rearing of barnacle larvae, well over one thousand cultures of larvae were initiated and studied with progressively increasing success. Early attempts at rearing resulted in high rates of mortality at the lower naupliar stages, with

no survival to the cyprid. As techniques became refined (most especially with the introduction of non-metallic sieves to facilitate water changes in rearing vessels), and a better understanding of feeding and algae culture was attained, survival rates increased and complete larval metamorphosis to the cyprid stage resulted. To date, several hundred individual cultures have yielded cyprids; data for several representative cultures of four barnacles, Balanus amphitrite, B. eburneus, B. improvisus and B. trigonus are given in Appendix A, Table 2 through 5. The data are presented in such a way as to indicate the number of days required to reach a certain level of development in each larval population. Thus, the six columns of figures under "Naupliar Stages" indicate the number of days from hatching to each of the six (denoted by Roman numerals) naupliar stages. Under "Cyprid Stage", data are presented on the number of days from hatching to first appearance of cyprids, and also on the number of days to produce larval populations consisting of 20%, 40%, 60%, and 80% cyprids. These data for the four barnacle species are summarized, also in Table 1.

TABLE 1

| SUMMARY OF REARING RATES FOR BARNACLE LARVAE | | |
|--|-------------------------|---------------------------|
| Species | Elapsed Time from Hatch | |
| | Cyprids First Appear | Cyprids 80% of Population |
| Balanus amphitrite | 10 to 14 days | 14 days |
| Balanus eburneus | 7 to 12 days | 9 to 17 days |
| Balanus improvisus | 8 to 14 days | 12 to 20 days |
| Balanus trigonus | 9 days | 11 to 13 days |

DISCUSSION

17. The techniques, described herein, developed by the Naval Applied Science Laboratory for the culturing of barnacle larvae are currently being used to mass-rear presettled barnacle cyprids in the laboratory in connection with the development of a bio-assay technique for studying marine antifouling agents. Careful searches of the technical literature of the biological sciences and of antifouling coatings technology indicate that this accomplishment represents (1) the first successful mass-rearing to the cyprid stage of larvae of barnacles Balanus amphitrite, B. eburneus, B. improvisus, and B. trigonus, (2) the first mass rearing of barnacle larvae at a laboratory whose location is distinctly removed from the barnacle's natural growing site; thus complying with the requirement that the methodology be operational independent of the barnacle's natural environment, and (3) the first rearing technique to provide for the ultimate use of the reared barnacle larvae in a methodology involving controlled settlement and attachment of cyprids upon test panels of antifouling coatings.

18. The availability of quantities of barnacle cyprids in a controlled environment provides the basis for a wide variety of new research attacks upon the old problem of fouling. Heretofore, antifouling studies have been carried out with relatively hit-and-miss, non-reproducible, protracted methods, which involve, for the most part, examination of adult barnacle (or other fouling) populations upon test panels which have been exposed on harbor test rafts. Investigations involving the actual settlement and attachment of barnacles have been limited, thus far, to occasional studies of the physiology and morphology of barnacle larvae and the sequence of changes incident to cyprid attachment. In this connection, Bernard and Lane (15) describe the attachment and decortication of the barnacle Balanus amphitrite niveus which were collected from natural sources during the planktonic naupliar and cyprid stages.

19. The problems involved in rearing and maintaining quantities of cyprids, and the difficulties of working with live cyprids (16) have, up until now, precluded the possibility of exploring fully the crucial area of the actual attachment of juvenile barnacles upon antifouling test panels. Now, with the establishment of the techniques described herein for the culture of barnacle cyprids, the following benefits will accrue: (1) investigations of antifouling toxics and antifouling toxic-containing coatings can be pursued with increased technical control and reproducibility, and with greater speed, (2) studies which heretofore were carried out on a small scale with limited results; such as studies of cyprid settlement and attachment, and the shell-building of young adult barnacles; can be expanded to yield information of great value for antifouling control, and (3) research, heretofore not attempted, in the area of bio-physical approaches to fouling control; such as the effect upon juvenile barnacles of acoustical energy, magnetic and electrical fields, light, and other stimuli or repellants; will

be facilitated. It is considered (17) that this development of techniques for the controlled mass-rearing of barnacle larvae to the cyprid stage, constitutes a breakthrough in the field of marine biology.

CONCLUSIONS

20. The following conclusions are drawn from the techniques described and the data presented herein:

a. Barnacle larvae of species Balanus amphitrite, B. eburneus, B. improvisus, and B. trigonus may be successfully mass-reared, from fertile ova to cyprid, in the laboratory.

b. Adult, ova-bearing barnacles, may be transported from their natural site to a laboratory at a distinctly removed geographical location and serve to provide embryos for successful laboratory culturing of larvae.

c. Nauplii of barnacles B. amphitrite, B. eburneus, B. improvisus, and B. trigonus can be maintained in vigorous, healthy, metamorphosing cultures upon a diet of the centric diatom Cyclotella nana.

d. The use of non-metallic, bolting-cloth sieves of the proper mesh size for the periodic changing of seawater in barnacle larvae rearing vessels, provides an efficient, workable means for maintaining healthy, vigorous larval cultures.

e. Using rearing techniques developed at this Laboratory, cultures consisting of populations, at least 80% of which have metamorphosed to the cyprid stage, may be attained as follows:

- (1) Balanus amphitrite: 14 days
- (2) Balanus eburneus: 9 to 17 days
- (3) Balanus improvisus: 12 to 20 days
- (4) Balanus trigonus: 11 to 13 days

CURRENT AND FUTURE WORK

21. Work on this task, currently in progress or planned for the near future, includes the following:

a. The laboratory culturing of an arctic species of barnacle, Balanus balanoides, whose habitate extends to local (New York harbor) waters, is proceeding successfully and a report on the mass-rearing of cyprids of this species will be prepared by May 1965.

b. Emphasis in the current laboratory work is being placed upon the development of techniques and standards for the controlled settlement and attachment of cyprids upon test surfaces.

c. A study of methods by which antifouling coatings may be laboratory-aged; and studies of means for correlating accelerated laboratory aging (or leaching) with natural aging are underway.

d. Plans are being made to develop methods for the controlled, laboratory mass-rearing of other fouling organisms. A probable choice for a second organism for an eventual complex laboratory fouling environment will be a species of tube worm.

e. Ultraviolet irradiation equipment for the treatment of incoming Laboratory seawater, modelled after equipment described in (18), has been built and is being installed.

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APPENDIX A

DATA ON DEVELOPMENT OF BARNACLE LARVAE

The data on Laboratory growth rates for four species of barnacle larvae are presented the following Tables:

Table 2 - Balanus amphitrite

Table 3 - Balanus obunus

Table 4 - Balanus improvisus

Table 5 - Balanus trigonus

TABLE 2

| DEVELOPMENT OF <u>BALANUS AMPHITRITE</u> LARVAE TO CYPRID STAGE | | | | | | | | | | | | | |
|---|-----------------------------|-------------------------------------|---|--------|--------|--------|---------|--------------|------------------|-----------------------|-----|-----|-----|
| Rear- ing Vessel No. | Embryo Maturity Group | Number of Larvae (Approx.) | Time (Days) from Hatch to Larval Stages | | | | | | | | | | |
| | | | Naupliar Stages | | | | | Cyprid Stage | | | | | |
| | | | I | II | III | IV | V | VI | First Appear. | Percent of Population | | | |
| | | | | | | | | | | 20% | 40% | 60% | 80% |
| L-4 | | 200 | <1 | 1 to 2 | 2 to 4 | 4 to 5 | 5 to 6 | 6 to 11 | 11 | | 17 | | |
| L-6 | | 200 | <1 | 1 to 2 | 2 to 5 | 5 to 6 | 6 to 7 | 7 to 11 | 11 | 12 | 15 | | |
| L-5 | | 200 | <1 | 1 to 2 | 2 to 4 | 4 to 9 | 9 to 10 | 10 to 14 | 14 | 15 | | | |
| L-10 | | 200 | <1 | 1 to 2 | 2 to 4 | 4 to 5 | 5 to 6 | 6 to 11 | 11 | | | | |
| L-11 | | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 5 | 5 to 6 | 6 to 11 | 11 | 12 | 15 | | |
| L-12 | | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 6 | 6 to 7 | 7 to 11 | 11 | 15 | | | |
| L-30 | 2 | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 5 | 5 to 7 | 7 to 10 | 10 | 11 | 12 | 13 | 14 |
| L-31 | 2 | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 5 | 5 to 7 | 7 to 11 | 11 | 11 | 12 | 14 | |

NOTE: Tables 2 through 5 - the absence of data entries indicates that no observations were made.

TABLE 3

| DEVELOPMENT OF <u>BALANUS EBURNEUS</u> LARVAE TO CYPRID STAGE | | | | | | | | | | | | | |
|---|-----------------------------|-------------------------------------|---|--------|--------|---------|---------|---------|------------------|-----------------------|-----|-----|-----|
| Rear- ing Vessel No. | Embryo Maturity Group | Number of Larvae (Approx.) | Time (Days) from Hatch to Larval Stages | | | | | | Cyprid Stage | | | | |
| | | | Naupliar Stages | | | | | | First Appear. | Percent of Population | | | |
| | | | I | II | III | IV | V | VI | | 20% | 40% | 60% | 80% |
| L-1 | | 200 | <1 | <1 | 1 to 2 | 3 to 4 | 5 | 6 to 8 | 8 | 10 | 13 | | |
| L-2 | | 200 | <1 | <1 | 1 to 2 | 3 to 4 | 5 | | 9 | | 16 | | |
| L-3 | | 200 | <1 | <1 | 1 to 2 | 3 to 4 | 5 | 7 to 8 | 9 | | | | 13 |
| L-7 | | | <1 | <1 | 1 to 2 | 2 to 4 | 5 to 11 | 6 to 12 | | | | | |
| L-8 | | | <1 | <1 | 1 to 2 | 2 to 4 | | | 12 | | | | |
| L-9 | | | <1 | <1 | 1 to 2 | 2 to 11 | 6 to 11 | 6 to 11 | 12 | 15 | | | |
| L-15 | | | <1 | <1 | 1 | | | | | | | | |
| L-16 | | | | | 1 | | | | | | | | |
| L-17 | | | | | 1 | | | | | | | | |
| L-26 | 3 | 1000 | <1 | 1 to 2 | 2 to 4 | 4 to 5 | 5 to 7 | 7 to 10 | 10 | 13 | 14 | | |
| L-27 | 3 | | <1 | 1 to 2 | 2 to 4 | 4 to 5 | 5 to 7 | 7 to 10 | 10 | | | | |
| L-28 | 3 | | <1 | 1 to 2 | 2 to 3 | 4 to 5 | 5 to 7 | 7 to 10 | 10 | | | | |
| L-29 | 2 | 600 | <1 | 1 to 2 | 2 to 4 | 4 to 5 | 5 to 7 | 7 to 9 | 10 | 12 | 13 | 14 | 16 |
| L-33 | 1 | 550 | <1 | <1 | 2 | 3 to 4 | 5 to 7 | 6 to 9 | 9 | 10 | 11 | 13 | 15 |
| L-35 | 1 | 200 | <1 | 2 | 2 | 3 to 6 | 4 to 7 | 7 to 9 | 10 | | 11 | 13 | |
| L-36 | 1 | 200 | <1 | 1 to 2 | 1 to 3 | 4 to 5 | 5 to 6 | 6 to 7 | 8 | 10 | 11 | | |
| L-37 | 1 | 750 | <1 | 1 to 2 | 1 to 3 | 3 to 4 | 4 to 7 | 6 to 7 | 8 | 11 | 13 | | |
| L-38 | 1 | 200 | <1 | 1 to 2 | 1 to 2 | 3 to 4 | 4 to 7 | 6 to 7 | 8 | 10 | | | |
| M-11 | 1 | 600 | <1 | 1 to 2 | 2 to 4 | 4 to 5 | 5 to 8 | 8 to 11 | 11 | 12 | 13 | 14 | 15 |
| M-12 | 2 | 250 | <1 | 1 to 2 | 2 to 3 | 3 to 4 | 4 to 8 | 8 to 11 | 11 | 13 | 14 | 15 | 17 |
| M-15 | 3 | 500 | <1 | 1 to 2 | 2 to 3 | 3 to 4 | 4 to 6 | 6 to 9 | 8 | | | | 9 |
| M-16 | 3 | 300 | <1 | 1 to 2 | 2 to 3 | 3 to 5 | 5 to 6 | 6 to 9 | 8 | | | | 9 |
| M-17 | 3 | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 4 | 4 to 6 | 6 to 9 | 8 | | 9 | 10 | 9 |
| M-21 | 2 | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 4 | 4 to 5 | 5 to 7 | 7 | 8 | | | 9 |
| M-22 | 2 | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 4 | 4 to 5 | 5 to 8 | 8 | | | | 9 |

TABLE 4

| DEVELOPMENT OF <u>BALANUS IMPROVISUS</u> LARVAE TO CYPRID STAGE | | | | | | | | | | | | | |
|---|-----------------------------|-------------------------------------|---|--------|--------|--------|---------|----------|------------------|-----------------------|-----|-----|-----|
| Rear- ing Vessel No. | Embryo Maturity Group | Number of Larvae (Approx.) | Time (Days) from Hatch to Larval Stages | | | | | | | | | | |
| | | | Naupliar Stages | | | | | VI | First Appear. | Cyprid Stage | | | |
| | | | I | II | III | IV | V | | | Percent of Population | | | |
| | | | | | | | | | | 20% | 40% | 60% | 80% |
| L-25 | | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 4 | 4 to 7 | 7 to 8 | 8 | 9 | 10 | 11 | |
| L-32 | 2 | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 5 | 5 to 6 | 6 to 10 | 10 | 10 | 11 | | 12 |
| L-34 | 2 | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 4 | 4 to 8 | 8 to 10 | 10 | 12 | | | 13 |
| M-2 | 1 | 600 | <1 | 1 to 2 | 2 to 4 | 4 to 5 | 5 to 12 | 12 to 14 | 14 | | | 19 | 20 |

TABLE 5

| DEVELOPMENT OF <u>BALANUS TRIGONUS</u> LARVAE TO CYPRID STAGE | | | | | | | | | | | | |
|---|-----------------------------|-------------------------------------|---|--------|--------|--------|--------|--------|------------------|---------------------------------------|----|-----|
| Rear- ing Vessel No. | Embryo Maturity Group | Number of Larvae (Approx.) | Time (Days) from Hatch to Larval Stages | | | | | | | | | |
| | | | Naupliar Stages | | | | | | First Appear. | Cyprid Stage Percent of Population | | |
| | | | I | II | III | IV | V | VI | | | | |
| | | | | | | | | | | | | 20% |
| L-19 | | 200 | <1 | 1 to 2 | 2 to 3 | 3 to 5 | 5 to 6 | 6 to 9 | 9 | | 10 | 11 |
| L-20 | | 200 | <1 | 1 to 2 | 2 to 4 | 4 to 6 | 6 to 7 | 7 to 9 | 9 | 10 | 11 | 13 |
| L-21 | | 200 | <1 | 1 to 2 | 2 to 4 | 4 to 6 | 6 to 7 | 7 to 9 | 9 | 10 | 12 | 13 |
| L-22 | | 200 | <1 | 1 to 2 | 2 to 4 | 4 to 6 | 6 to 7 | 7 to 9 | 9 | | 10 | 11 |

APPENDIX B

PHOTOMICROGRAPHS OF BARNACLE LARVAE

The eight pages following, consist of four series of photomicrographs, taken in vivo and reproduced here to a uniform scale, of the larval stages of four species of barnacle. The series are as follows:

Figures 1A and 1B - Balanus amphitrite

Figures 2A and 2B - Balanus eburneus

Figures 3A and 3B - Balanus improvisus

Figures 4A and 4B - Balanus trigonus

Each series of photographs represents a complete record of the larval stages of each species; from fertilized ova, through the six naupliar stages, to the planktonic cyprid stage. The appearance of these photographs in this report represents the first publication of complete photographic records of the larval stages of the barnacle.

BALANUS AMPHITRITE

LARVAL STAGES

0 100 200 300
SCALE: MICRONS



FERTILIZED OVA



FIRST STAGE NAUPLIUS



SECOND STAGE NAUPLIUS



THIRD STAGE NAUPLIUS

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Figure 1A - Balanus amphitrite, Larval Stages.

PHOTO L-19901-1

BALANUS AMPHITRITE

LARVAL STAGES

0 100 200 300
SCALE: MICRONS



FOURTH STAGE NAUPLIUS



FIFTH STAGE NAUPLIUS



SIXTH STAGE NAUPLIUS



PLANKTONIC CYPRID STAGE

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Figure 1B - Balanus amphitrite, Larval Stages.

PHOTO L-19901-2

BALANUS EBURNEUS

LARVAL STAGES

0 100 200 300
SCALE: MICRONS



FERTILIZED OVA



FIRST STAGE NAUPLIUS



SECOND STAGE NAUPLIUS



THIRD STAGE NAUPLIUS

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Figure 2A - Balanus eburneus, Larval Stages.

PHOTO L-19901-3

BALANUS EBURNEUS

LARVAL STAGES

0 100 200 300
SCALE: MICRONS



FOURTH STAGE NAUPLIUS



FIFTH STAGE NAUPLIUS



SIXTH STAGE NAUPLIUS



PLANKTONIC CYPRID STAGE

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Figure 2B - Balanus eburneus, Larval Stages.

PHOTO L-19001-4

**BALANUS IMPROVISUS
LARVAL STAGES**

0 100 200 300
SCALE : MICRONS



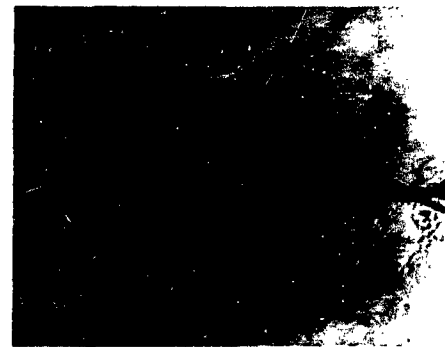
FERTILIZED OVA



FIRST STAGE NAUPLIUS



SECOND STAGE NAUPLIUS



THIRD STAGE NAUPLIUS

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Figure 3A - Balanus improvisus, Larval Stages.

PHOTO I-19901-5

BALANUS IMPROVISUS LARVAL STAGES

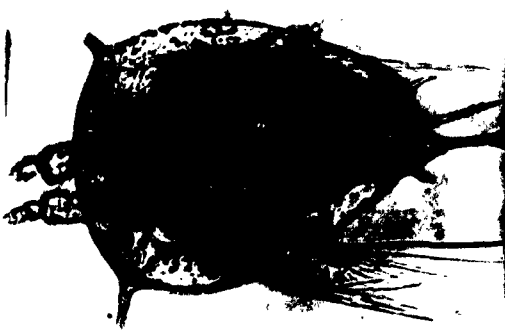
0 100 200 300
SCALE : MICRONS



FOURTH STAGE NAUPLIUS



FIFTH STAGE NAUPLIUS



SIXTH STAGE NAUPLIUS



PLANKTONIC CYPRID STAGE

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Figure 3B - Balanus improvisus, Larval Stages.

PHOTO L-19901-6

BALANUS TRIGONUS LARVAL STAGES

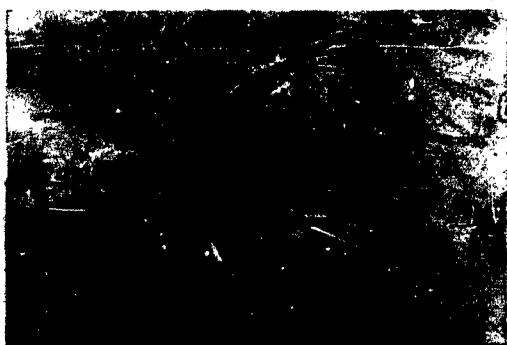
0 100 200 300
| | | |
SCALE: MICRONS



FERTILIZED OVA



FIRST STAGE NAUPLIUS



SECOND STAGE NAUPLIUS



THIRD STAGE NAUPLIUS

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Figure 4A - Balanus trigonus, Larval Stages.

PHOTO L-19901-7

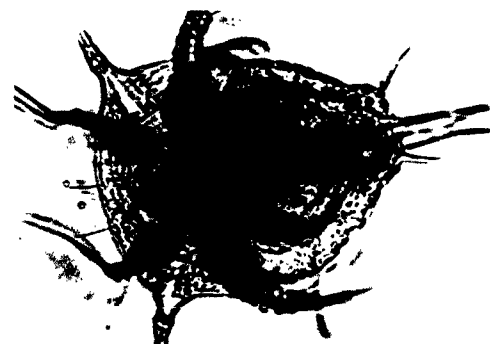
BALANUS TRIGONUS

LARVAL STAGES

0 100 200 300
SCALE: MICRONS



FOURTH STAGE NAUPLIUS



FIFTH STAGE NAUPLIUS



SIXTH STAGE NAUPLIUS



PLANKTONIC CYPRID STAGE

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Figure 4B - Balanus trigonus, Larval Stages.

PHOTO L-19901-8

APPENDIX CMETHODS FOR IDENTIFICATION OF ADULT BARNACLES

The Table below briefly outlines the identification of the five species of adult barnacles currently being studied at the Laboratory.

| IDENTIFICATION OF ADULT BARNACLES | | | | |
|-----------------------------------|----------------|--------------------------|--|--|
| Species | Color | Surface Texture | Markings | Other Characteristics |
| Balanus balanoides (Note a) | white | chalky flaky rough | | No basal plate. |
| B. eburneus | ivory | smooth lustrous | | Shell plates have wide radii. |
| B. amphitrite | white | smooth | Purple-brown longitudinal striations on shell plates. | Shape of opercular opening: rhombic |
| B. improvisus | white to ivory | smooth lustrous | Faintly pigmented longitudinal striations on shell plates. | Shell plates have narrow radii. |
| B. trigonus | pink | rugose | | Shape of opercular opening: triangular |

Note a: Rearing of Balanus balanoides not reported herein.

APPENDIX DMETHODS FOR CULTURING ALGAE

The following is a brief outline of the procedures employed at the Laboratory for growing quantities of the centric diatom Cyclotella nana for use as food for barnacle larvae cultures:

1. Inoculums - Inoculums of diatom cells are supplied to the Laboratory as non-sterile transfers of algae stock culture. They are stored, in viable condition in the Laboratory's incubator at a temperature of 65F.

2. Culturing algae - The following list indicates the sequence of successive steps in the culturing process. Each successive culture is inoculated with the cells cultured in the previous step.

| <u>Step</u> | <u>Volume</u> |
|--------------------|---------------|
| 1. Inoculum | |
| 2. Starter Culture | 10 ml |
| 3. Small Flask | 100 ml |
| 4. Large Flask | 1 l |
| 5. Carboy | 10 l |

When the concentration of cells in the carboys reaches approximately 1.5×10^6 cells per milliliter, the culture is ready for use.

3. Enrichment media - Two enrichment media solutions are added, one milliliter of each per liter of seawater, to algae cultures. The compositions of the solutions are as follows:

Solution A

| | |
|----------------------------------|-----------|
| NaH ₂ PO ₄ | 20.0 g |
| HCl | 0.2 g |
| Biotin | 0.001 g |
| Vitamin B ₁₂ | 0.001 g |
| Pyridoxin HCl | 0.1 g |
| Calcium pantathionate | 0.2 g |
| Distilled water | 1.0 liter |

Solution B

| | |
|--------------------|-----------|
| NaNO ₃ | 150.0 g |
| NH ₄ Cl | 50.0 g |
| Ferric sequestrine | 10.0 g |
| Distilled water | 1.0 liter |

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4. Cell counts - The cell densities of algae cultures are determined with a hemacytometer. This instrument, ordinarily used for determining blood-cell counts, provides a means for counting, under the microscope, the number of cells in a fixed volume of culture solution. From this count, the overall cell density can be calculated.



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Figure 5 - Lamella, Consisting of Hundreds of Embryos, Being Removed from
Body of Parent Barnacle.

PHOTO L-19901-9



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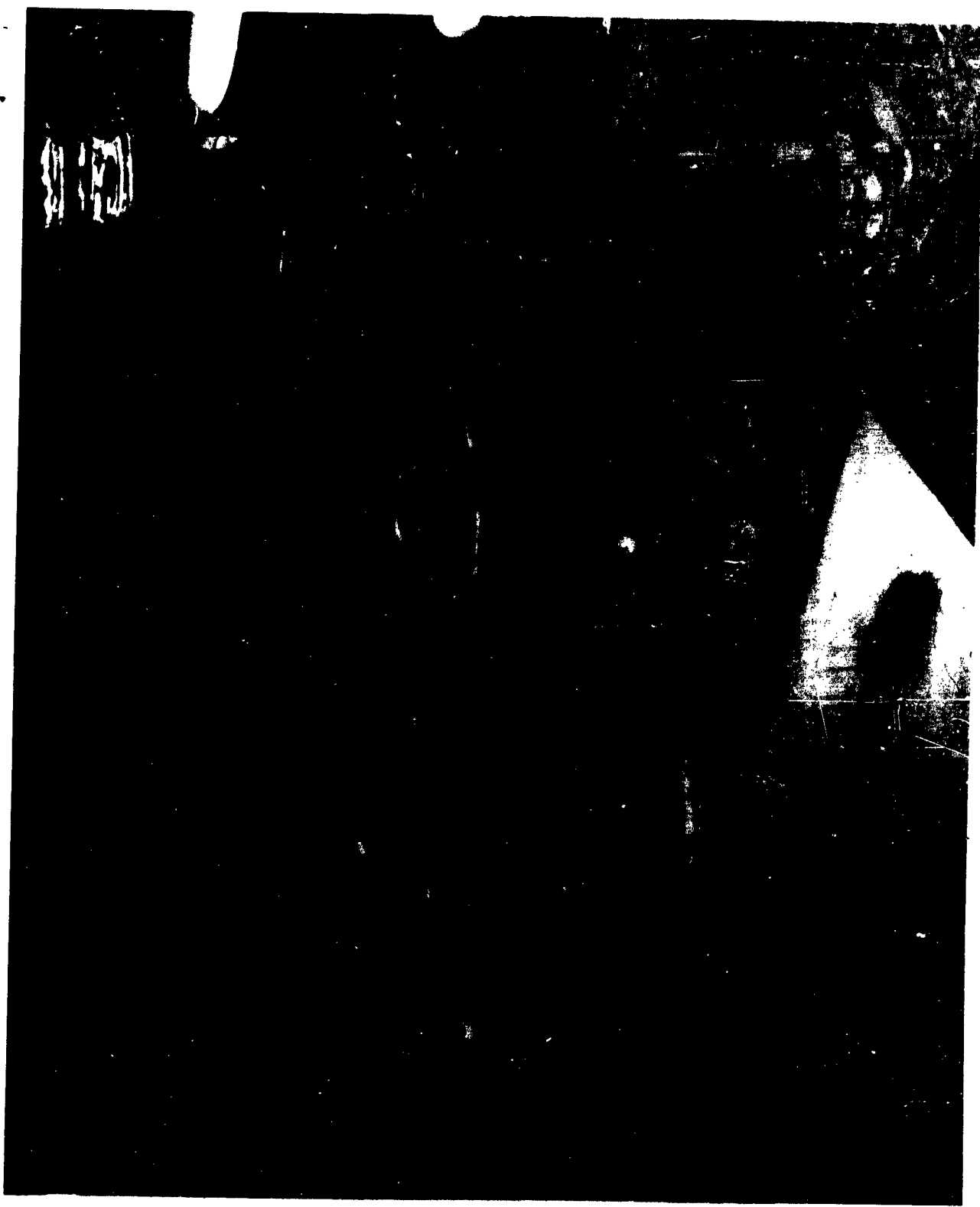
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Figure 6 - Non-Metallic Bolting-Cloth Sieves

In Front: Sieve Components-Paper Hoops, Bolting Cloth, and Wax.

In Rear : Finished Sieves

PHOTO L-19901-10



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PHOTO L-19901-11

Figure 7 - Laboratory Set-up for Transferring and Counting Nauplii.

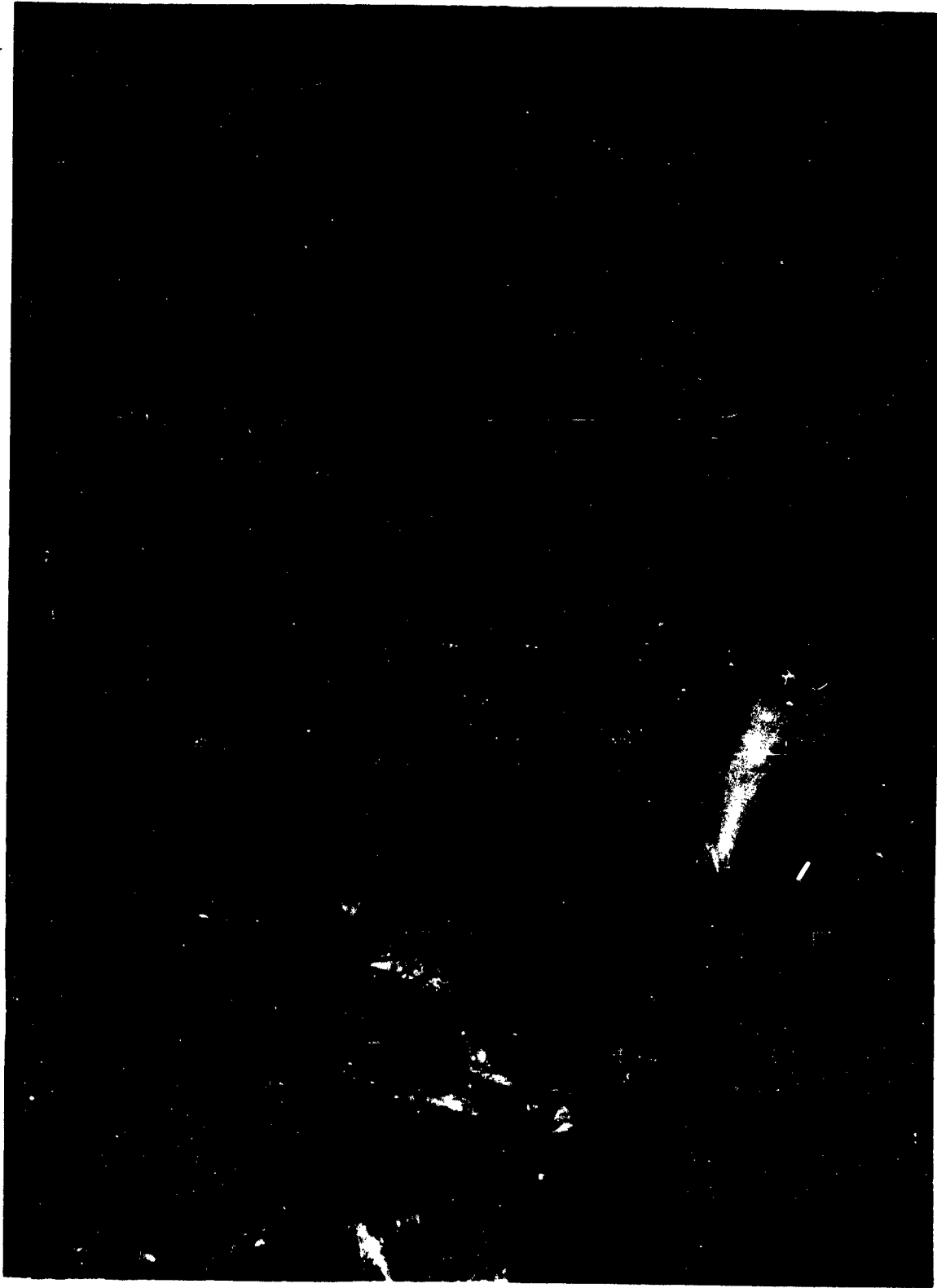


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Figure 8 - Close-up of Nauplius Collecting Vessel. Beam from Spotlight, Traversing Seawater, Causes Phototropic Nauplii to Concentrate Nearest the Light Source.

PHOTO L-19901-12



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Figure 9 - Laboratory Set-up showing Nine Rearing Vessels in which Nauplii are Reared to Cyprid Stage. In foreground: An Extra Settlement Petri Dish.

PHOTO L-19901-13



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Figure 10 - A Population of Larvae being Passed, from Rearing Vessel,
through a Bolting-Cloth Sieve.

PHOTO L-19901-114



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Figure 11 - A Population of Larvae being Flushed from Sieve into a
Shallow Examination Dish,

PHOTO L-19901-15

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| 13. ABSTRACT Studies of marine fouling and evaluations of antifouling coatings and methods have been carried on at field stations under naturally occurring conditions. A more aggressive, fundamental study of the marine-fouling problem can be attempted, and evaluations of antifouling materials can be accomplished with greater speed and regularity, if studies employing live marine organisms are conducted under laboratory-controlled conditions. The techniques which, for the first time, succeeded in the laboratory mass-rearing of four species of acorn barnacles indigenous to the U. S. Atlantic coastline are set forth. The methods for removal of embryos from parent barnacles, feeding, and maintenance of proper environment for larval stages are described. Methods are described by which fertile barnacle ova are hatched, and metamorphose with successive ecdyses through six free-swimming naupliar stages, to the swimming-and-crawling cyprid stage. Photomicrographs of barnacle embryos, the six naupliar larval stages, and the presettled cyprid larval stage are presented. The future capabilities of this laboratory methodology and its anticipated contribution toward the development of improved anti-fouling materials are discussed. | | |

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|---|--------|----|--------|----|--------|----|
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